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Negative regulation of the thyroid-stimulating hormone α gene by thyroid hormone: Receptor interaction adjacent to the TATA box

(glycoprotein hormones/*erbA* protooncogene/gene transcription/steroid receptors)

V. KRISHNA K. CHATTERJEE, JENN-KUEN LEE, ANNE RENTOUMIS, AND J. LARRY JAMESON*

Thyroid Unit, Massachusetts General Hospital, Harvard Medical School, Boston, MA 02114

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ABSTRACT **Thyroid-stimulating hormone α and β subunit genes are negatively regulated by thyroid hormone at the transcriptional level. Transient gene expression studies were used to demonstrate that the erbAβ form of the thyroid hormone receptor mediates negative regulation of the α-subunit promoter in a hormone-dependent manner. In JEG-3 choriocarcinoma cells, which are deficient in thyroid hormone receptors, coexpression of erbAβ with αCAT reporter genes markedly suppressed αCAT expression after treatment with thyroid hormone, whereas a reporter gene containing a known positive thyroid response element was induced. Thus, a single form of thyroid hormone receptor mediates both positive and negative responses to thyroid hormone in this system. Transient expression analyses of α gene 5′ flanking sequence deletion mutants localized the negative thyroid response element to the proximal region of the promoter between −100 and +4 base pairs. The location of the negative thyroid response element in the α gene is therefore distinct from that of previously identified regulatory elements including the tissue-specific upstream regulatory elements, the cAMP response elements, and the glucocorticoid response elements. Overlapping segments of the α promoter were examined for potential thyroid hormone receptor binding sites by using gel shift assays and biotinylated DNA-binding studies. A specific thyroid hormone receptor binding site was identified between −22 and −7 base pairs, which is immediately downstream from the TATA box. This region of the α promoter interacts with erbAβ receptor synthesized *in vitro* as well as with endogenous nuclear thyroid hormone receptors, and it competes for receptor binding to a known positive thyroid response element. These studies suggest a mechanism for negative regulation in which the thyroid hormone receptor interacts with the α gene promoter immediately downstream of the TATA box to inhibit transcription.**

Thyroid hormones exert a myriad of physiological effects, including modulation of growth and development, as well as regulation of a variety of pathways of intermediary metabolism (1). The effects of thyroid hormone (triiodothyronine; T3) are mediated by activation of receptors that stimulate or repress the transcription of specific genes (2). T3 receptors are structurally related to the viral oncogene v-*erbA* and, together with the steroid hormone, vitamin D, and retinoic acid receptors, constitute a superfamily of proteins sharing sequence homology. Comparison of the structures of these receptors reveals several functional domains that include one or more transcriptional activating domains, a cysteine-rich DNA-binding domain that interacts with specific DNA sequences in target genes, and a carboxyl-terminal ligand-binding domain (2). Two major groups of T3 receptors, classified as α and β forms, have been identified, and there are structural variants within each group (3). The functional significance of the different forms of T3 receptors is not understood.

The DNA sequences that mediate transcriptional activation or repression in response to T3 are referred to as thyroid response elements (TREs). The TRE sequences bind T3 receptors with high affinity (4, 5) and have been studied extensively by using the rat growth hormone (rGH) promoter as a model of positive regulation (1). Negative regulation by T3 has also been demonstrated for a number of genes (6, 7), but the DNA response elements that confer negative regulation have not been fully defined.

Thyroid-stimulating hormone (TSH) is a pituitary glycoprotein hormone that stimulates the thyroid gland to synthesize and secrete T3. TSH is a heterodimer composed of α and β subunits that are encoded by separate genes. In classic feedback fashion, T3 suppresses TSH secretion (8) and gene transcription (9). The kinetics of T3 action correlate with receptor occupancy and do not require new protein synthesis, suggesting that the activated receptor acts directly to negatively regulate TSH α and β subunit gene expression (9).

In view of these observations, we used transient expression analyses to examine the 5′ flanking region of the human TSH α gene for negative TREs. Receptor binding sequences within the α gene were delineated by using either *in vitro* translated receptor protein or endogenous receptor in GH4 cell nuclear extracts.

MATERIALS AND METHODS

Plasmid Constructions. Construction of −846 αCAT, which contains 846 base pairs (bp) of 5′ flanking sequence and 44 bp of exon I linked to the gene encoding chloramphenicol acetyltransferase (CAT), has been described (10). The 5′ deletion mutants of −846 αCAT were generated by using exonuclease III digestion, and the endpoints were confirmed by DNA sequencing. Heterologous constructions were made in which the 18-bp direct repeat cAMP response elements (CREs) from the α promoter were inserted upstream of either the −100-bp α promoter or the −109-bp thymidine kinase promoter. A fragment containing the 72-bp repeats from the simian virus 40 (SV40) enhancer was inserted into a *Bam*HI site upstream of −171 and −100 αCAT. Additional CAT vectors include TreTKCAT (provided by D. Moore, Boston), which contains two copies of a TRE derived from the rGH gene (11) inserted upstream of the thymidine kinase promoter (pTK14A multimer), and GAPDHCAT (provided by M. Alexander-Bridges, Boston), which contains −487 to +20 bp of the human glyceraldehyde-3-phosphate dehydrogenase

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Abbreviations: T3, triiodothyronine; TSH, thyroid-stimulating hormone; CRE, cAMP response element; TRE, thyroid response element; rGH, rat growth hormone; CAT, chloramphenicol acetyltransferase; ABC, avidin–biotin complex; SV40, simian virus 40.

*To whom reprint requests should be addressed at: Thyroid Unit, Bulfinch B, Massachusetts General Hospital, Boston, MA 02114.

promoter (12). RSVerbAβ (provided by H. Samuels, New York) (13) is an expression vector that contains the human erbAβ form of the T3 receptor cDNA (14) driven by the Rous sarcoma virus promoter. The receptor cDNA in this vector was subcloned into M13mp18, and a single amino acid change (Cys-122 to Ser) was made by site-directed mutagenesis (15). All plasmid DNAs were purified by two cycles of CsCl density gradient centrifugation.

Cell Culture and Transient Expression Assays. JEG-3 cells (HTB 36, American Type Culture Collection) were grown in Dulbecco's modified Eagle's medium containing 10% (vol/vol) fetal calf serum, penicillin (100 units/ml), and streptomycin (100 μg/ml). For transient expression assays, triplicate plates of cells were transfected by a 4-hr exposure to a calcium phosphate/DNA precipitate (16) followed by culturing in medium containing 10% charcoal-stripped fetal calf serum with or without 1 nM T3. After 36 hr, cellular extracts were assayed for CAT activity (17) by using aliquots diluted to maintain enzyme activities within the linear range of the assay. CAT activity is expressed relative to −846 αCAT, which was included as a standard in each experiment to correct for variations in transfection efficiency. Measurements of T3 receptors in JEG-3 cells were made following mock transfection with carrier plasmid or transfection with RSVerbAβ. Receptor binding was assayed by labeling cells in culture with [^{125}I]T3 to measure specific binding to isolated nuclei as well as by performing Scatchard analyses of *in vitro* binding of [^{125}I]T3 to nuclear receptors (13).

Receptor–DNA Binding Assays. Human erbAβ DNA (provided by R. Evans, San Diego, CA) was transcribed *in vitro* using T7 polymerase (14). Capped transcripts were used to program reticulocyte lysates as suggested by the manufacturer (Promega Biotec). Avidin–biotin complex (ABC) DNA-binding assays were performed as described by Glass *et al.* (4, 5). Overlapping 40-bp regions of the α gene promoter between −132 bp and +29 bp were synthesized as complementary duplex oligonucleotides containing identical 5′ overhangs to allow incorporation of biotin-11-dUTP residues (5). For example, including 5′ overhangs (lowercase letters), the −22 to −7 bp oligonucleotide consists of 5′ aaggggatccG-CAGGTGAGGACTTCA 3′ on the coding strand and 5′ aggaagatctTGAAGTCCTCACCTGC 3′ on the noncoding strand. Oligonucleotide pairs were annealed, filled in with biotin-11-dUTP (Enzo Biochemicals), dATP, dCTP, and dGTP by using Klenow polymerase, gel-purified, and quantitated by fluorometry. Biotinylated DNA (200 fmol) was incubated with 50,000–80,000 trichloroacetic acid-precipitable cpm of [^{35}S]methionine-labeled erbAβ receptor for 40 min at 24°C. The receptor–DNA complexes were precipitated with streptavidin-agarose beads (Bethesda Research Laboratories) and quantitated by scintillation spectrometry. Alternatively, nuclear extracts were prepared from GH4 cells as described previously (5) and used in gel mobility shift assays that contained 10,000 cpm of ^{32}P-labeled TRE DNA (≈0.5 fmol) from the rGH gene (−186 to −162 bp), 4 μg of poly[d(I-C)], 20 μg of GH4 extract protein, and 500 fmol of various competitor DNAs. The 3′ recessed ends of the oligonucleotides were filled in with unlabeled nucleotides before use in gel shift assays. Protein-bound DNA complexes were resolved from unbound DNA by nondenaturing polyacrylamide gel electrophoresis (5, 10, 18). In separate experiments, GH4 extracts were incubated with [^{125}I]T3 to label endogenous T3 receptors (5), and gel shift assays were subsequently performed with 20 fmol of various unlabeled DNA fragments.

RESULTS

Inhibition of αCAT Expression in JEG-3 Choriocarcinoma Cells Requires Cotransfection of the erbAβ Receptor and Is Hormone Dependent. T3 treatment of native JEG-3 cells, or cells mock transfected with a Rous sarcoma virus expression vector for β-galactosidase, did not inhibit the activity of transiently expressed αCAT fusion genes or stimulate the expression of a construction that contains known positive TREs linked to the thymidine kinase promoter (Fig. 1*A Left* and 1*B Left*). To test the hypothesis that this unresponsiveness to T3 was due to a deficiency or absence of T3 receptors, receptor binding assays were performed. These assays showed that native JEG-3 cells exhibit minimal [^{125}I]T3 nuclear binding (12 fmol per 100 μg of DNA). However, after transfection of a T3 receptor expression vector (RSVerbAβ), specific [^{125}I]T3 binding increased to 110 fmol per 100 μg of DNA with a K_d of 0.4 nM.

Concordant with the ability to bind [^{125}I]T3, αCAT expression was negatively regulated by 75–80% and TreTKCAT expression was stimulated 10-fold in cells transfected with RSVerbAβ and treated with T3 (Fig. 1*A Center* and 1*B Center*). The activities of control constructions such as GAPDHCAT (Fig. 1*C*) or TKCAT (see Fig. 3*B*) were unaffected by T3 treatment. Thus, expression of erbAβ in JEG-3 cells converts them to a receptor-positive phenotype in which the same form of activated receptor mediates both positive and negative regulation by T3. Dose–response experiments using various amounts of cotransfected receptor or added T3 indicated that maximal inhibition of αCAT activity occurred with 250 ng of cotransfected RSVerbAβ and 1 nM T3.

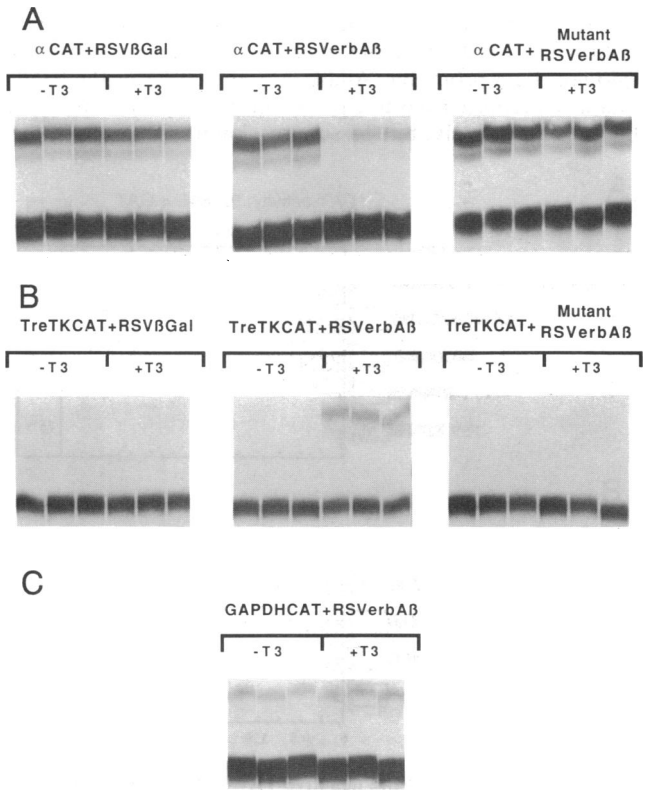

FIG. 1. Suppression of α gene expression requires the T3 receptor and activation by T3. Autoradiograms of CAT assays from triplicate transfections are illustrated. JEG-3 choriocarcinoma cells were transfected with 5 μg of −846 αCAT (*A*) or TreTKCAT (*B*) reporter plasmids together with 250 ng of the indicated Rous sarcoma virus expression vectors. (*C*) GAPDHCAT is a control reporter plasmid. RSVβGal expresses β-galactosidase. RSVerbAβ expresses the β form of the T3 receptor, and the mutant RSVerbAβ contains a serine substitution for cysteine at residue 122 in the DNA-binding domain. Cells were treated without (−T3) or with (+T3) 1 nM T3 for 36 hr, and CAT enzyme activity was measured.

Subsequent experiments were therefore performed under these conditions.

A mutant erbAβ receptor was constructed in which a cysteine residue (Cys-122) in the "zinc finger" region of the DNA-binding domain was converted to serine by using site-directed mutagenesis. When tested by cotransfection in JEG-3 cells, this mutant was nonfunctional with regard to activation of TreTKCAT as well as suppression of αCAT (Fig. 1*A Right* and 1*B Right*). Thus, both positive and negative regulation by the T3 receptor in this context requires an intact DNA-binding domain.

Localization of the Negative TRE to −100 bp of the α Promoter by Using Deletional Analyses. A series of 5′ flanking sequence deletion mutants were studied to delineate the region of the human α promoter that mediates negative regulation by T3. Deletion of 5′ flanking sequence between −846 and −244 bp did not affect either basal activity or the degree of suppression (78–86%) by T3 (Fig. 2*A*). Likewise, deletion of the 5′ untranslated sequence between +4 and +44 bp in several of these constructions also failed to alter the degree of T3 suppression (V.K.K.C., unpublished data). Deletions downstream of −244 bp remove known strong positive regulatory sequences in the α gene (10, 19, 20). Thus, when upstream regulatory elements were deleted (e.g., −171 αCAT or −159 αCAT) or when one or both copies of the CREs (−146 to −111 bp) were removed (e.g., −132 αCAT or −100 αCAT), there was a marked reduction in basal activity, but inhibition by T3 was maintained (Fig. 2*B*).

Because basal activity is low after removal of the α gene enhancer elements, heterologous constructions were used to confirm the location of the negative TRE in the proximal region of the α promoter. Addition of the CREs or the SV40 enhancer increased basal α promoter activity by 8-fold and 60-fold, respectively, but T3 suppression was retained in the presence of either enhancer (Fig. 3*A*). Insertion of the SV40 enhancer upstream of −171 αCAT, a construction that contains the CREs and one of the upstream regulatory elements, resulted in slightly greater inhibition (68%) by T3, suggesting that these α gene regulatory elements may enhance the degree of inhibition by T3. In contrast to the α promoter, addition of the CREs to a heterologous thymidine kinase promoter failed to confer regulation by T3, whereas a known positive TRE was fully active with this promoter (Fig. 3*B*). These observations are consistent with localization of the negative TRE in the α gene within the first 100 bp of the α promoter.

Delineation of T3 Receptor Binding Sites in the α Promoter. In view of the transient expression data suggesting that a negative TRE resides in the downstream region of the α promoter, we performed receptor binding studies to determine whether receptor–DNA interactions also occur within this region. Sequences in the α gene between −132 and +29 bp were synthesized as overlapping 40-bp fragments to include known regulatory elements such as the CRE, the CAAT box, and the TATA box as well as regions such as exon I that do not contain known regulatory DNA sequences. Initially, these α gene fragments were tested for their ability to interact with the T3 receptor in ABC DNA-binding assays (Fig. 4) (4, 5). [^{35}S]Methionine-labeled human erbAβ receptor was prepared by *in vitro* transcription and translation and was incubated with biotinylated DNA sequences followed by precipitation of DNA–receptor complexes using streptavidin-agarose beads. Specific binding of the *in vitro* translated receptor to α gene sequences was compared with that to a known T3 receptor binding sequence from the rGH gene

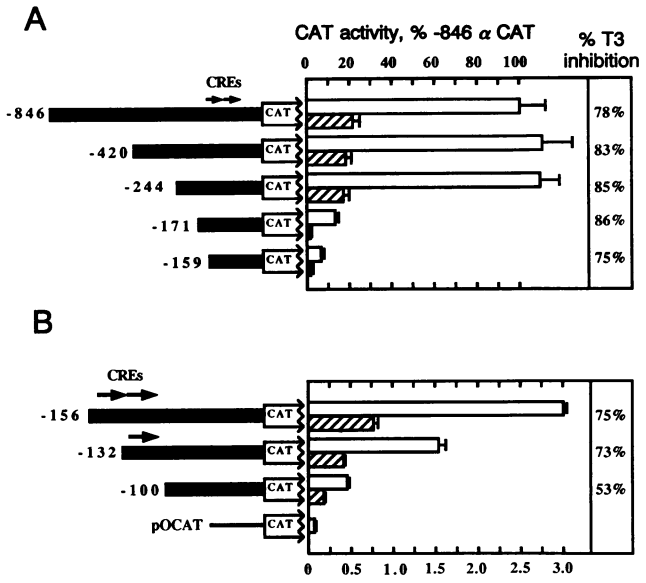

FIG. 2. Delineation of the negative TRE in the α gene. Deletion mutants of αCAT were cotransfected into JEG-3 cells with RSVerbAβ as described in Fig. 1. Deletions of the α gene 5′ flanking sequence are illustrated schematically to the left of the bar graphs. The location of the repeated copies of the CREs are indicated by arrows. CAT activity (mean ± SD) relative to −846 αCAT is shown without (open bars) or with (hatched bars) treatment with 1 nM T3. The percentage of inhibition by T3 is shown at the right. The basal activity of −846 αCAT ranged between 1.5 and 3.0% conversion per μg of protein per hr in different experiments. The activity of pOCAT, a promoterless reporter plasmid, is shown for comparison. (*A*) Deletions from −846 to −159 bp. (*B*) Deletions from −156 to −100 bp (note the different scales on the ordinates).

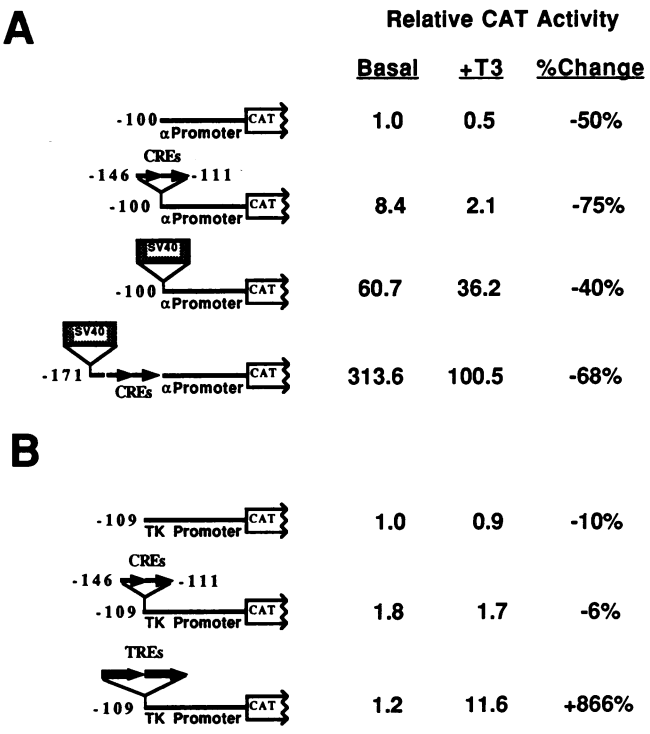

FIG. 3. Localization of the α gene TRE by using heterologous constructions. The indicated regulatory elements (CREs, the SV40 72-bp repeat enhancer, and TREs) were inserted upstream of the α promoter (*A*) or thymidine kinase (TK) promoter (*B*). The heterologous constructions were cotransfected into JEG-3 cells with RSVerbAβ in the absence or presence of T3 as described in Fig. 1. CAT activity is expressed relative to the truncated promoters without additional regulatory elements. The basal activities of the −100 αCAT and TKCAT were 0.01 and 0.08% conversion per μg of protein per hr, respectively.

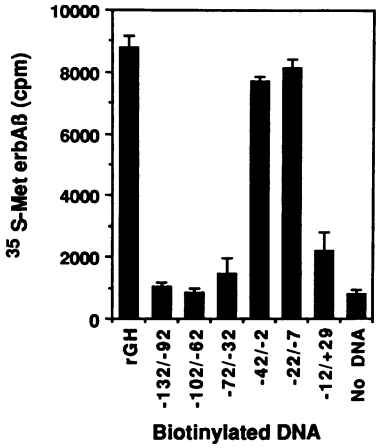

FIG. 4. T3 receptor binding to the α gene promoter sequences. ABC DNA-binding assays of *in vitro* translated, [^{35}S]methionine-labeled erbAβ receptor to various biotinylated DNA sequences were performed and the results are expressed as the mean ± SD of triplicate assays. The sequences from the α promoter (excluding the biotinylated linkers) are shown below each bar. rGH refers to the −186 to −162 bp fragment of the rGH promoter.

(−186 to −162 bp). As described previously (4), this rGH TRE bound ^{35}S-labeled T3 receptor effectively (Fig. 4). Among the overlapping α promoter fragments, only the region between −42 and −2 bp exhibited substantial receptor binding. This region of the α promoter is notable for the presence of a canonical TATA box sequence between −29 and −26 bp, located in a characteristic position upstream from the transcriptional start site. The sequence determinants for binding to the −42 to −2 bp region were defined further by demonstrating that binding was equally effective with a 16-bp fragment that extends from −22 to −7 bp (Fig. 4). Thus, the TATA box element itself is not required for receptor recognition, and a region of the α gene promoter between −22 and −7 bp is sufficient for receptor binding. A similar profile for receptor binding to the α promoter fragments in the ABC DNA-binding assay was obtained by using [^{125}I]T3-labeled endogenous receptors present in GH4 cell nuclear extracts (V.K.K.C., unpublished data).

As an independent measure of receptor interactions with the α gene promoter, gel shift binding assays were performed with nuclear extracts of GH4 cells (5). T3 receptors in nuclear extracts were labeled with [^{125}I]T3 and then incubated with unlabeled DNA. Receptor–DNA complexes were identified by the location of ^{125}I-labeled T3 after electrophoresis through polyacrylamide gels (Fig. 5*A*). In the absence of DNA, T3-labeled proteins do not enter the gel efficiently (no DNA lane). A single [^{125}I]T3-labeled complex was identified by using either unlabeled rGH TRE sequence (−186 to −162 bp) or the −42 to −2 bp α gene fragment. There was minimal interaction of T3-labeled proteins with the other α gene fragments. In separate experiments, the −22 to −7 bp fragment from the α promoter was also shown to bind the T3 receptor in the gel shift binding assay.

The rGH TRE was labeled with ^{32}P and used in gel shift assays to identify specific DNA–receptor complexes in unlabeled extracts of GH4 cells (Fig. 5*B*). Four DNA–protein complexes were formed, and one of these (band 3) comigrated with the [^{125}I]T3-labeled complex (Fig. 5*A*). Excess unlabeled rGH TRE competed specifically for binding to band 3 as well as for binding to several other proteins. Of the five different α gene competitor sequences examined, only the −42 to −2 bp fragment competed specifically for binding to band 3. Thus, both the positive TRE from the rGH gene and the putative negative TRE in the α gene compete for binding to the T3 receptor.

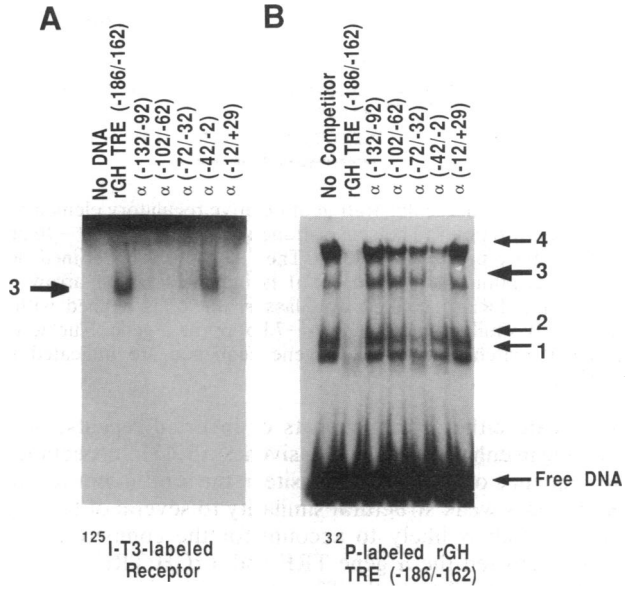

FIG. 5. Gel mobility shift assays of T3 receptor–DNA complexes. (*A*) Binding of [^{125}I]T3-labeled proteins in GH4 nuclear protein extracts to various DNA fragments. The indicated unlabeled DNA fragments were incubated with [^{125}I]T3-labeled GH4 extracts, and protein–DNA complexes were resolved by nondenaturing polyacrylamide gel electrophoresis. (*B*) Competition for binding to a TRE from the rGH gene (−186 to −162 bp) by α promoter fragments. The ^{32}P-labeled rGH TRE was incubated with GH4 nuclear extract protein in the presence of a 1000-fold molar excess of the indicated competitor DNA fragments. Free DNA and protein-bound DNA complexes (indicated by arrows labeled 1–4) were resolved by nondenaturing polyacrylamide gel electrophoresis run in parallel with the experiment shown in *A*.

DISCUSSION

Previous studies have shown the existence of several distinct regulatory DNA elements in the 5′ flanking region of the α gene (10, 19, 20). Upstream regulatory elements that interact with placental-specific factors involved in basal transcription are located between −185 and −150 bp. Two identical copies of CREs are found between −146 and −111 bp and bind nuclear factor CREB (21). In addition, the glucocorticoid receptor has been shown to bind to domains that overlap with the CREs, and negative regulation of the α gene by glucocorticoids has been proposed to involve interference with the binding or transcriptional activity of CREB (22). Deletion analyses demonstrate that the negative TRE in the α gene resides within a 104-bp region of the α promoter between −100 and +4 bp (Fig. 2).

Because further deletions of the α promoter reduced the level of expression below that which can be reliably analyzed for negative regulation, we examined overlapping segments of the α promoter for possible receptor binding sites to further localize the TRE. These studies identify a single locus for receptor binding between −22 and −7 bp. The demonstration in gel shift assays that the DNA–protein complex formed with both the rGH TRE and the α TRE (band 3 in Fig. 5*B*) is also labeled by [^{125}I]T3 (Fig. 5*A*) provides further evidence that this sequence binds T3 receptor. It is likely that this receptor binding site in the α promoter is involved in the negative regulation observed in the transient expression assays.

Although several groups have examined the DNA sequence determinants for T3 receptor binding and responsiveness in the rGH promoter, no clear consensus sequence has yet emerged. In fact, there appears to be redundancy in this promoter with several distinct elements that can bind T3 receptor (18) and mediate functional activity (1). Based upon these analyses, TREs have been derived by using mutations

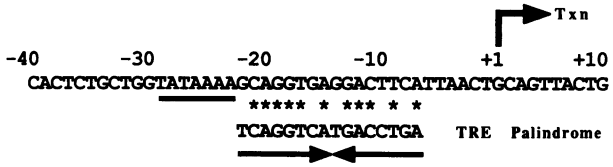

FIG. 6. Schematic illustration of putative regulatory elements in the α gene promoter. The sense strand sequence between -40 and $+10$ bp of the α promoter is shown. The TATA box is underlined, and the transcriptional start site (Txn) is indicated by an arrow. A palindromic TRE described by Glass *et al.* (5) is aligned with a structurally similar region (-22 to -7 bp) of the α gene. Nucleotide sequence matches with the α gene sequence are indicated by asterisks.

that create either direct repeats or inverted repeats, often resulting in enhanced T3 responsiveness (5, 11). Inspection of the sequence of the T3 binding site in the α promoter reveals that it bears weak structural similarity to several of the rGH TREs, which is likely to account for the competition observed between the α gene TRE and a rGH TRE (-186 to -162 bp) for binding to the T3 receptor (Fig. 5*B*). The α TRE is most similar (11 of 16 bp) to a palindromic TRE described by Glass *et al.* (5) in which a 3-bp deletion between the two halves of a consensus sequence for the estrogen response element was found to create a powerful T3 receptor binding site and response element (Fig. 6).

The mechanisms by which gene transcription is negatively regulated are not well understood. Protein–protein interactions have been suggested as a mechanism for transcriptional inhibition of AP-2 mediated expression by the SV40 large tumor antigen (23) and for inhibition of the prolactin gene expression by the estrogen receptor (24). Alternatively, distinct proteins could compete for binding to a common target DNA site to result in negative regulation. Examples of such a mechanism include a CCAAT box displacement protein that binds to a site that overlaps the CCAAT box in the histone H2B promoter (25) and glucocorticoid receptor interference at CRE binding sites in the α gene (22). The observation that a mutation in the DNA-binding domain of the T3 receptor precludes negative regulation suggests that interaction of the receptor with DNA sequences is required to suppress α promoter transcription.

The demonstration that a binding site for the T3 receptor exists between the TATA box and the transcriptional initiation site in the α gene promoter provides a potentially powerful mechanism for transcriptional inhibition. Recent studies of the adenovirus major late promoter have shown that the formation of a transcription complex with RNA polymerase II involves the interaction of several transcription factors to form a stable DNA–protein complex overlying the TATA box and extending beyond the transcriptional start site (26). It is tempting to speculate that T3 receptor occupancy of this locus in the α promoter may preclude the binding of the TATA box factor or other associated transcription factors to inhibit formation of an active transcription complex. Studies using purified transcription factors and *in vitro* transcription assays will be required to examine this mechanism in greater detail.

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